

Characterization of the Effects of Divalent Cations on the Coupled Activities of the H⁺-ATPase in Tonoplast Vesicles

ABSTRACT

The substrate requirement of the H⁺-ATPase in purified corn root tonoplast vesicles was investigated. The coupled activities, ATP hydrolysis and proton pumping, were simultaneously supported only by Mg²⁺ or Mn²⁺. The presence of Ca²⁺ or Ba²⁺ did not significantly affect the coupled activities. The addition of Cd²⁺, Co²⁺, Cu²⁺, and Zn²⁺ inhibited both the hydrolysis of Mg-ATP and the proton transport. However, the inhibition of proton pumping was more pronounced. Based on equilibrium analysis, both ATP-complexed and free forms of these cations were inhibitory. Inhibition of the hydrolysis of Mg-ATP could be correlated to the concentrations of the ATP-complex of Zn. On the other hand, the free Cu²⁺ and Co²⁺ were effective in inhibiting hydrolysis. For proton pumping, the ATP complexes of Co²⁺, Cu²⁺, and Zn²⁺ were effective inhibitors. However, this inhibition could be further modulated by free Co²⁺, Cu²⁺, and Zn²⁺. While the equilibrium concentrations of Cd-ATP and free Cd²⁺ were not estimated, the total concentration of this cation needed to inhibit the coupled activities of the H⁺-ATPase was found to be in the range of 10 to 100 micromolars. The presence of free divalent cations also affected the structure of the lipid phase in tonoplast membrane as demonstrated by the changes of emission intensity and polarization of incorporated 1,6-diphenyl-1,3,5-hexatriene. The differential inhibition caused by these cations could be interpreted by interactions with the protogenic domain of the membrane as previously proposed in "indirect-link" mechanism.

Based on the studies of isolated membrane vesicles, the tonoplast of plant cells has been shown to contain two inwardly directed, electrogenic H⁺-pumps, one powered by ATP and the other by PPi (1, 2, 6, 14). The proton electrochemical potential, $\Delta\mu_{H^+}$, which contains a ΔpH (acidic inside) and a $\Delta\Psi$ (positive inside) generated by these pumps serves as the driving force for the secondary transport of other solutes across tonoplast (3, 16, 17). The tonoplast H⁺-ATPase requires Mg-ATP as the substrate and can be inhibited by nitrate ions (7, 19). The properties of this enzyme, *e.g.* vanadate insensitivity, anion dependence, and the generation of proton electrochemical potential, are similar to the Mg-ATPase associated with isolated vacuoles (29).

Unlike the plant plasma membrane H⁺-ATPase, which contains only one polypeptide (molecular mass \approx 100 kD) and forms a phosphorylated intermediate during catalysis (5, 15), the tonoplast H⁺-ATPase is composed of at least three different peptide subunits and does not involve phosphorylation of the enzyme in the reaction pathway (4, 11). While the

exact molecular process leading to proton pumping remains to be established, the kinetics of proton gradient generation by the H⁺-ATPase in tonoplast vesicles has been studied. Based on initial rate measurements, a fixed stoichiometry of 2 H⁺/ATP was reported for the tonoplast ATPase of red beet microsomal membranes (1). This observation is consistent with the notion that the proton pumping is directly coupled to ATP hydrolysis in the tonoplast system. It implies that the proton pumping must share at least part of the exact molecular pathway leading to ATP hydrolysis, as described by the classical chemiosmotic model (12). However, using a detailed kinetic model for the proton movement, we found that certain treatments did not change the H⁺ leakage of the membrane but yielded differential effects on the coupled activities in highly purified tonoplast membrane vesicles (27, 28). This result suggested that the coupling between proton pumping and ATP hydrolysis may be indirect, as proposed earlier (25).

The kinetic scheme used in our tonoplast experiments was originally developed to describe the energy-dependent proton movement in chloroplasts (9), mitochondria (22), and purple membrane system (23). Recently, based on pure curve-fitting techniques, an empirical expression for the proton movement in plant membrane vesicles was advanced (10). While this empirical expression has been independently developed, it is interesting to note that, as will be shown later in this report, the empirical expression is mathematically equivalent to our kinetic model.

In spite of these advances, relatively few studies have been made on the effects of multivalent cations on the coupled function. Although it has been shown in two separate reports that Mg-ATP is the preferred substrate for either ATP hydrolysis (8) or proton pumping (7), the kinetic origin of this preference remains to be established. Furthermore, the possible direct interactions of multivalent cations with the H⁺-ATPase have never been systematically investigated. In view of the probable important role of the proton translocating enzyme in regulating the transport processes of tonoplast membrane, a thorough understanding on the possible interaction between the enzyme and multivalent cations is needed. In this report, we studied the ATP hydrolysis and its associated proton pumping using a variety of metal-ATP complexes to establish the kinetic origin of the substrate preference of corn root tonoplast H⁺-ATPase. We also investigated the influence of multivalent cations on Mg-ATP-dependent enzyme activities to establish the interactions between metal ions and the membrane. The results confirmed the absolute requirement of Mg-ATP or Mn-ATP as the substrate for both the hydroly-

ysis of ATP and the proton pumping activities. The presence of inhibitory cations and their ATP complexes always resulted in a preferential inhibition of the proton pumping process. This differential effect supports our previously proposed "indirect coupling" between ATP hydrolysis and proton pumping in corn root tonoplast system (27, 28).

MATERIALS AND METHODS

Isolation of Tonoplast Membrane Vesicles

Corn seeds (*Zea mays* L. cv FRB 73, Illinois Foundation Seeds¹) were germinated and harvested as previously described (26). Approximately 60 to 80 g fresh weight of excised roots were homogenized in a chilled mortar with a precooled pestle at 4°C in 0.3 M sucrose solution (3 mL/g of roots) containing 5 mM EDTA, 5 mM 2-mercaptoethanol, 5 mM DTE, and 0.1 M Hepes, pH 7.7 (adjusted at 4°C). The crude homogenate was filtered through four layers of cheesecloth. Differential centrifugation at 6,000g for 20 min and 80,000g for 35 min was then employed to obtain a microsomal pellet. Microsomes were resuspended in 20 to 25 mL of homogenizing medium and centrifuged again at 80,000g for 35 min. Washed microsomes were suspended in grinding medium and overlaid on a 15% to 45% (w/w) linear sucrose gradient buffered with 5 mM Hepes-Mes, pH 7.7, plus 1 mM DTE and centrifuged at 4°C for 16 to 18 h at 84,000g (average) in a Beckman SW-28 rotor as described previously (27). The tonoplast fractions (between 19% and 23% sucrose), which were >90% pure as judged by nitrate inhibition, were pooled and used for experiments.

ATP Hydrolysis Measurement

ATP hydrolysis catalyzed by the H⁺-ATPase was assayed by the direct measurement of inorganic phosphate released at 22°C as previously described (26). The appearance of inorganic phosphate was determined in a volume of 2.2 mL of the basal solution containing 50 mM KCl, 7.5 μM acridine orange, 17.5 mM Mes, pH 6.45, tonoplast vesicles, and different concentrations of multivalent cations as specified in the text. The reaction was started by the addition of substrate (ATP complexes) and was terminated by ice-cold TCA at appropriate time intervals during which the ATP hydrolysis was constant. The amount of inorganic phosphate released was determined by the malachite green-molybdate-phosphate complex formation (24). Within employed concentration range, the divalent cations did not affect the complex formation.

Measurement of Proton Movement

In our previous reports (27, 28), we found that the proton movement associated with tonoplast vesicles could be described by assuming that the net proton pumping rate (flux), as determined by the absorbance change of acridine orange at 492 nm, obeyed the equation:

$$d\delta/dt = R_0 - k_1\delta \quad (1)$$

¹ Reference to brand and firm does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

in which $d\delta/dt$, R_0 , k_1 , and δ represented the net proton transport rate at time t after the addition of ATP, the initial proton pumping rate, a rate constant measuring membrane leakage and other possible inhibition processes such as back pressure (30) and pump slippage (13), and the extent of proton transport at time t , respectively. At steady state, the net transport rate reached zero. Thus:

$$R_0 = k_1\delta_s \quad (2)$$

in which δ_s was the extent of proton transport at the steady state. Eq. 2 was integrated to:

$$\delta = \delta_s(1 - \exp(-k_1t)) \quad (3)$$

or

$$\ln(1 - \delta/\delta_s) = -k_1t \quad (4)$$

Since the proton transport was expressed by the absorbance change, Eq. 3 could be rewritten as:

$$(A_t - A_i) = (A_s - A_i)(1 - \exp(-k_1t))$$

or

$$A_t = A_s - \Delta A \exp(-k_1t) \quad (5)$$

in which A_i , A_t , and A_s represented the absorbance of the indicator at time 0, t , and steady state, respectively. If fluorescence change was used to measure proton movement, then Eq. 5 assumed the form of:

$$F_t = F_s - \Delta F \exp(-k_1t) \quad (6)$$

The equation developed by empirical curve fitting (Eq. 2 in ref. 14) had an identical mathematical expression as shown here in Eq. 6. We had demonstrated in our previous works (27, 28) that, under employed experimental conditions, the ATP hydrolysis remained constant during the build-up of the proton gradient in corn root tonoplast vesicles. Thus, the initial proton pumping rate was related to the constant ATP hydrolysis rate by:

$$R_0 = mR_{ATP} \quad (7)$$

The quantity " m " would then represent the stoichiometric ratio of H⁺/ATP. Experimentally, the proton movement was qualitatively measured by the absorbance change of acridine orange at 492 nm at 22°C. After obtaining the proton pumping information (1–2 min after reaching steady state), the same samples were used to determine the rate of ATP hydrolysis. A typical set of observed time course of proton pumping under various experimental conditions is shown in Figure 1. The growth phase (before reaching steady state) of proton pumping was fitted by Eq. 4 to determine k_1 . The R_0 was then calculated by Eq. 2. It should be mentioned that the spectroscopic response of acridine orange to pH changes was not affected by the presence of the divalent cations (data not shown).

Calculation of ATP Complex Concentration

The dissociation constants of divalent cation-ATP complexes determined in neutral pH solution at 25°C by Taqui Kahn and Martell (20) are listed in Table I. In the presence

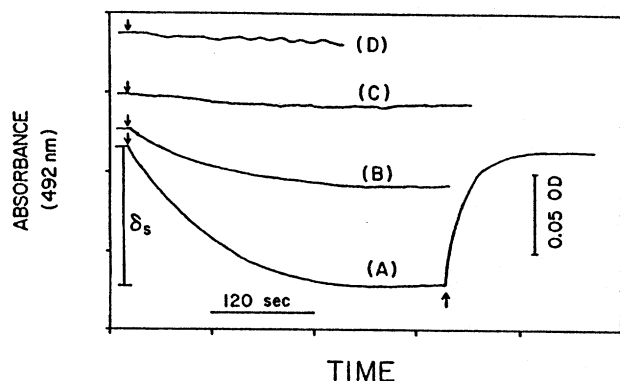
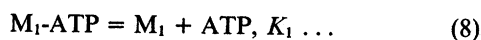


Figure 1. Proton pumping of tonoplast vesicles. The kinetic traces of proton pumping were recorded by the absorbance change of acridine orange as described. Purified tonoplast vesicles were incubated in the proton pumping medium at 22°C for 10 min before the addition of substrate as indicated by down arrows (↓). Trace (A) represents proton pumping resulted from the addition of 2 mM Mg and ATP and the discharge of proton gradient by the presence of 0.2 μM CCCP (↑). Trace (B) shows the effect of including 60 μM Cd²⁺ in (A) during incubation. Trace (C) indicates the result of using 1 mM Mg, 1 mM Cd, and 2 mM ATP as the substrate. Trace (D) exhibits the effect of adding 2 mM Cd and ATP as substrate.

of two different divalent cations, the concentrations of ATP complexes and free cations were estimated from the following simultaneous equilibria:



in which K_1 and K_2 represented the dissociation constants. With the total concentrations of added M_1 , M_2 , and ATP as a , b , and c mM, respectively, the ratio of Eq. 8 and 9 yielded:

$$\frac{[M_2\text{-ATP}](a - [M_1\text{-ATP}])}{[M_1\text{-ATP}](b - [M_2\text{-ATP}])} = K_1/K_2 \dots \quad (10)$$

To solve Eq. 10, the concentrations of $M_1\text{-ATP}$ and $M_2\text{-ATP}$ under the experimental conditions were first roughly estimated by assuming that the other cation was absent. Thus:

$$[M_1\text{-ATP}] + [M_2\text{-ATP}] = d(\text{variable}) \dots \quad (11)$$

Quadratic solution of Eq. 10 and 11 yielded the refined concentration terms needed to calculate the apparent dissociation constants, $K_1(a)$ and $K_2(a)$. The value of $d(\text{variable})$ was then adjusted further and the calculation process repeated until the percentage of errors of the calculated apparent dissociations were within a reasonable range ($\pm 0.1\%$) from the literature values.

Fluorescence Polarization Measurement

The effect of multivalent cations on the structure of the tonoplast membrane was qualitatively estimated from the change in fluorescence properties of DPH² incorporated in the membrane (18). The tonoplast vesicles suspended in the basal medium without acridine orange were treated with 1.90

μM DPH for 20 min at 22°C. The suspension was then illuminated with vertically polarized light (370 nm) and the fluorescence intensities at the parallel (I_1) and the perpendicular (I_2) directions were measured at 424 nm with a Perkin-Elmer MPF44A Spectrofluorimeter. The polarization was calculated by the ratios of $(I_1 - I_2)/(I_1 + I_2)$. The effects of the cation were determined by including 0.2 mM ion in the incubation medium before the measurement.

Protein Concentration Measurement and Others

The protein content of the tonoplast membrane vesicles was determined by a modified Lowry method using bovine serum albumin as standard (27). All the chemicals and biochemicals were obtained from commercial sources and were of analytical grades. The chloride salts of divalent cations were used in this study.

RESULTS

Requirement of Divalent Cation for H⁺-ATPase Activities

Previous studies indicated that the anion-sensitive ATP hydrolysis in a corn root microsomal fraction (8) and the proton pumping in oat root tonoplast vesicles (7) preferred Mg-ATP as the substrate. Furthermore, other divalent cations, with the exception of Mn²⁺, failed to stimulate either activity. We have re-examined this substrate requirement of the H⁺-ATPase in highly purified tonoplast vesicles of corn roots (Fig. 2). The enzyme exhibited negligible activities in the absence of divalent cation (less than 1% of that induced by Mg-ATP). However, rates of both ATP hydrolysis and proton pumping were strongly stimulated by the presence of either Mg²⁺ or Mn²⁺ (molar ratio of divalent cation/ATP = 1.0). With the same molar ratio to ATP, none of the other tested cations induced detectable proton pumping activity, although considerable ATP hydrolysis was observed with the presence

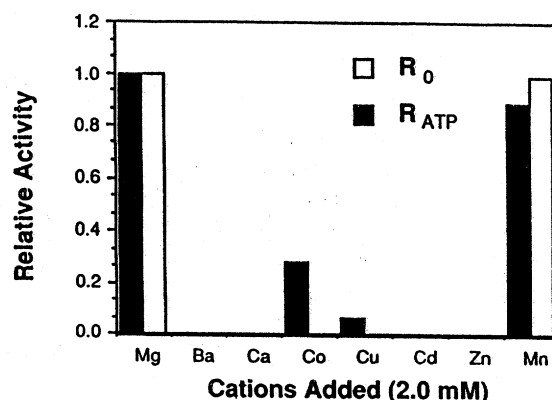


Figure 2. Divalent cation requirement of ATP hydrolysis and proton pumping by tonoplast vesicles. ATPase activities were assessed at 2 mM ATP with the addition of 2 mM each of tested cations. Initial rates of ATP hydrolysis (R_{ATP}) and proton transport (R_0) are represented by left and right bars, respectively. Activity is expressed relative to that in the presence of Mg, which corresponded to an activity of 351 nmol of Pi/min/mg of protein and 1.93 ΔA/min/mg of protein for ATP hydrolysis and proton pumping, respectively. The rates were calculated as specified in Materials and Methods.

² Abbreviation: DPH, 1,6-diphenyl-1,3,5-hexatriene.

of Co. Similarly, little, but measurable, ATP hydrolysis was induced by the presence of Cu. Thus, the tonoplast H⁺-ATPase of corn roots has an absolute requirement for Mg or Mn as the substrate for ATP-hydrolysis-supported proton pumping. It is interesting to note that the presence of certain cations, *e.g.* Co, induced ATP hydrolysis but not proton pumping.

Effects of Divalent Cations on H⁺-ATPase Activities

Divalent cations are known to form strong complexes with ATP at neutral pH (20) with the dissociation constants in the range of 10⁻² to 10⁻¹ mM (Table I). Under the experimental conditions mentioned in Figure 2, the extent of complexation ranged from 60.6% for Ba²⁺ to 94.9% for Cu²⁺. Since ATP itself did not support the enzyme activities, the results shown in Figure 2 strictly illustrated the preferred form of ATP complex for the corn root tonoplast H⁺-ATPase and did not provide any information concerning the possible interactions between the cations and the enzyme.

The following experiments were performed to evaluate reactions between divalent cations and the ATPase. Keeping Mg-ATP as substrate (2.5 mM Mg²⁺ and 2.0 mM ATP), the coupled reactions were measured in the presence and absence of other divalent cations (Table I). In this experiment, the ratio of Mg²⁺ to the added cations was kept at 5 to 1 to assure that most of the ATP was as Mg-ATP. An examination of Table I indicated that, under the experimental conditions, the effects of added divalent cations might be grouped into two classes. Class I cations that substantially inhibited the hydrolysis of Mg-ATP and essentially abolished the net proton pumping included Cu²⁺, Co²⁺, Cd²⁺, and Zn²⁺. Class II cations included Ca²⁺ and Ba²⁺, which exhibited certain measurable effects on ATP hydrolysis and proton pumping. The presence of Ba²⁺ slightly decreased the initial proton pumping rate (*R*₀) and slightly increased the membrane H⁺ leakage (*k*₁). On the other hand, Ca²⁺ increased the initial proton pumping rate

without any significant effect on the leakage. Consequently, the net extent of proton translocation at steady state (*δ*_s) was decreased by Ba²⁺ but increased by Ca²⁺. Since Class II cations did not significantly inhibit either proton pumping or ATP hydrolysis, only Class I cations were further investigated in this study.

Effective Form of Class I Cation in Inhibition

The results of Table I indicated that the presence of Class I cation significantly inhibited the coupled activities of the H⁺-ATPase. Furthermore, the proton pumping appeared to have a greater sensitivity to their presence. This preferential inhibition of proton pumping was not a consequence of an increase in membrane leakage, *k*₁. However, since both complexed (M-ATP) and free (M) cations were available in the equilibrium mixtures, it would be of interest to know the effective molecular form of the inhibitors.

For this purpose, the concentrations of Mg, M, and ATP were adjusted so that the concentration of M-ATP complex was increased (Table II) over the levels from the experiments in Table I. Although the concentrations of Mg-ATP were lowered by about 50%, they were still substantially higher than the *K*_m of the ATPase (0.1–0.2 mM). In general, the change in experimental conditions from Table I to Table II doubled [M-ATP] but decreased [M] by about 25% for Class I cations. Thus, the decrease in the inhibition of Mg-ATP hydrolysis observed with Co and Cu could be attributed to decreased concentration of the free cations. On the other hand, the increased inhibition of Mg-ATP hydrolysis by Zn could be attributed to an increase in [Zn-ATP]. While the quantitative analysis for Cd experiment was not possible for lack of exact value of dissociation constant of Cd-ATP, the results of Tables I and II qualitatively suggested that its effect on Mg-ATP hydrolysis was similar to that of Zn. The inhibitory effects of ATP complexes (Zn and Cd) might be attributed but not limited to the competitive binding at the catalytic site

Table I. Effects of Divalent Cations on the Activities of H⁺-ATPase

The total concentrations of Mg and ATP were 2.500 and 2.000 mM, respectively. The concentration of M added was 0.500 mM. This information was used to calculate the equilibrium concentrations of Mg-ATP, M-ATP, free Mg, free M, and free ATP. The *K*(Mg-ATP) and *K*(M-ATP) were apparent dissociation constants (in millimolars) derived from calculated concentrations. The literature *K*_{dis} (ref. 23) for Ba-ATP, Ca-ATP, Mg-ATP, Co-ATP, Mn-ATP, Zn-ATP, and Cu-ATP are 51.290, 10.720, 6.026, 2.188, 1.660, 1.413, and 0.741 × 10⁻² mM, respectively.

Cation (M)	[Mg-ATP]	[M-ATP]	[M]	<i>K</i> (Mg-ATP)	<i>K</i> (M-ATP)	<i>R</i> _{ATP}	<i>R</i> ₀	<i>k</i> ₁
<i>mM</i>								
None ^a	1.834	—	—	0.060	—	100	100	100
Ba	1.751	0.108	0.392	0.060	0.513	107	89.5	120
Ca	1.630	0.257	0.243	0.060	0.107	95.4	123	110
Co	1.506	0.403	0.097	0.060	0.022	16.5	0.00	—
Zn	1.482	0.431	0.069	0.060	0.014	29.4	0.00	—
Cu	1.456	0.460	0.041	0.060	0.007	3.67	0.00	—
Cd	—	—	—	—	—	29.4	0.00	—

^a The initial ATP hydrolysis rate (*R*_{ATP}), the initial proton pumping rate (*R*₀), and the membrane leakage (*k*₁) obtained in the presence of Mg and ATP were 312 nmol/min/mg of protein, 2.078 Δ A/min/mg of protein, and 0.716 min⁻¹, respectively. Those values were arbitrarily assigned as 100s to derive the relative activities (average of two measurements with probable error as 2%) obtained in the presence of added M.

Table II. Effects of Class I Divalent Cations on the H⁺-ATPase

The total concentrations of Mg and ATP used were 1.000 and 2.000 mM, respectively. The concentration of M added was 1.000 mM. This information was used to calculate the equilibrium concentrations of Mg-ATP, M-ATP, free Mg, free M, and free ATP. The $K(\text{Mg-ATP})$ and $K(\text{M-ATP})$ were calculated and compared to literature K_{dis} s. The percentage of error between the apparent and literature values was less than 0.1%.

Cation (M)	[Mg-ATP]	[M-ATP]	[M]	R_{ATP}	R_0
<i>mM</i>					
None ^a	0.946	—	—	100	100
Co	0.814	0.923	0.077	60.1	0.00
Zn	0.805	0.946	0.054	3.8	0.00
Cu	0.796	0.969	0.031	80.9	0.00
Cd	—	—	—	19.1	0.00

^a The initial ATP hydrolysis rate (R_{ATP}) and the initial proton pumping rate (R_0) obtained in the presence of Mg and ATP were 302 nmol of Pi/min/mg of protein and 2.03 $\Delta\text{A}/\text{min}/\text{mg}$ of protein, respectively. Those values were arbitrarily assigned as 100s to derive the relative activities (average of two experiments) obtained in the presence of added M.

for Mg-ATP in the ATPase. For Co and Cu, the binding of free cations at some other regulatory site(s) to induce the inhibition could not be discounted.

The results mentioned in Table II demonstrated again that the proton pumping of the tonoplast H⁺-ATPase was more sensitive to the presence of Class I cations. It should be mentioned that the presence of Ba and Ca under the conditions of Table II did not significantly alter the coupled activities of the enzyme (data not shown).

Effects of Class I Cations on Proton Pumping

The results described in Tables I and II demonstrated that Class I cations had a profound effect on the activities of the tonoplast H⁺-ATPase. Furthermore, we identified that both the ATP complexes (Zn and Cd) and the free ions (Co and Cu) might be the effective inhibitors to the hydrolysis of Mg-ATP. Since no proton pumping was observed, information on the effective inhibitory species of Class I cations to this activity could not be determined. The inhibition of proton pumping could originate from an enhancement of the membrane leakage (increase in k_1). This enhancement would be similar to the effect of protonophores (uncouplers), which could slightly increase the hydrolysis rate of Mg-ATP in corn root tonoplast vesicles (27). However, as shown in Tables I and II, Class I cations decreased the ATP hydrolysis rate and, therefore, might not act as protonophores under employed conditions.

To ascertain that an increase in membrane leakage (k_1) was not the primary cause of observed decrease in proton pumping, experiments mentioned in Table III were performed. In these experiments, the total concentrations of the cations were reduced to levels that did not cause significant inhibition to Mg-ATP hydrolysis. In addition, the experimental conditions were also adjusted to allow a significant change in the concentration of either free or ATP-complexed M but not both. As shown, the concentration of Mg-ATP was relatively constant.

The membrane leakage as measured by k_1 was either unchanged or decreased (less leakage). The proton pumping rate and, therefore, the coupling with ATP hydrolysis as measured by R_0/R_{ATP} , was also decreased. However, additional leakage, induced by the presence of higher concentrations of Class I cations (Table II), could not be ruled out.

Certain interesting points emerged when the data in Table III were examined closely. For all cations tested, a significant increase in [M-ATP] with only a minimal change in [M] resulted in a decrease in proton pumping (comparing B to A). Keeping [M-ATP] nearly unchanged (comparing C to B), a decrease in [M] resulted in less proton pumping with Cu and Co but measurable increase in proton pumping with Zn. These results suggest that (a) the ATP complexes of Cu, Zn, and Co may be the predominant inhibitors of the proton pumping process; (b) free Cu and Co may effectively displace their ATP complexes from the inhibition site and thus reduce the observed inhibition; and (c) free Zn binding to the membrane by displacing Zn-ATP or at additional site(s) may result in a greater inhibition of proton pumping.

Efficiency of Cd²⁺ Inhibition

Although the equilibrium concentrations of complexed and free Cd were not obtained, the effectiveness of Cd in inhibiting the coupled ATPase activities was clearly demonstrated in Tables I, II, and III. The potency of Cd inhibition was investigated further. Both the proton pumping rate and the ATP hydrolysis rate were very sensitive to Cd (Fig. 3). The pattern of differential inhibition was observed throughout the concentration range of added Cd²⁺. For example, the inclusion of 50 μM Cd²⁺ in the assaying medium caused a 60% inhibition of proton pumping rate but only decreased ATP hydrolysis rate by 10%. It should be mentioned that the observed decrease in initial proton pumping rate was not accompanied by an increase of membrane leakage (k_1). In fact, the value of k_1 decreased slightly as the concentration of Cd²⁺ increased (data not shown).

The efficiency of Cd²⁺ to inhibit the H⁺-ATPase activities was also evaluated by Michaelis-Menten kinetics (Fig. 4). The presence of Cd²⁺ did not change the linear relationship between $1/V$ versus $1/S$, suggesting that the basic feature of the molecular pathway leading to Mg-ATP hydrolysis and its associated proton pumping remained unchanged. A comparison between Figures 4A and 4B again indicated that the proton pumping process was more sensitive to Cd. The fact that the inhibition could be relieved by increasing Mg-ATP concentration indicated that the effective binding of Cd²⁺ (ATP-complexed or free) could be regulated by the interaction between Mg-ATP and the tonoplast H⁺-ATPase. The values of K_m for Mg-ATP hydrolysis and proton pumping in the absence of Cd²⁺ were determined as 0.11 and 0.26 mM, respectively (Fig. 4). This observation that the coupled processes could respond to Mg-ATP concentration differently was also observed in our previous work (27). Since the inhibition of ATP hydrolysis and proton pumping by Cd²⁺ did not appear to obey a simple competitive pattern, no attempt was made to estimate k_i values from the data shown in Figure 4. Such a complex mode of inhibition would suggest that existence of multiple binding sites for Cd²⁺ in the membrane. It

Table III. Effects of Class I Cations on Proton Pumping

In the absence of Class I cations (None), the total concentrations of Mg and ATP were 2.000 and 1.800 mM, respectively. In the experiments labeled as (A), these concentrations were 2.000 mM for Mg and 1.700 mM for ATP. In (B), they were 2.500 mM (Mg) and 1.700 mM (ATP). In (C), they were 2.000 and 1.850 mM for total Mg and ATP, respectively. These variations had negligible effects on the rates of ATP hydrolysis and proton pumping when inhibitory cations were absent.

The procedures used to determine the equilibrium concentrations were identical to those mentioned in Table I. The values of R_0 and R_{ATP} for None were 2.05 $\Delta A/\text{min}/\text{mg}$ of protein and 325 nmol of $\text{P}_i/\text{min}/\text{mg}$ of protein, and were assigned as 100 and 100, respectively.

Cation	[Mg-ATP]	[M-ATP]	[M]	k_1	R_0	R_0/R_{ATP}
	mm			min^{-1}		
None	1.576	—	—	1.021	100	1.00
Cu, 0.050 (A)	1.480	0.048	0.002	1.056	57.6	0.582
Cu, 0.034 (B)	1.567	0.032	0.002	1.019	89.5	0.923
Cu, 0.032 (C)	1.587	0.031	0.001	1.010	74.8	0.771
Zn, 0.110 (A)	1.443	0.101	0.009	0.920	90.4	0.913
Zn, 0.070 (B)	1.542	0.061	0.009	0.858	95.4	0.984
Zn, 0.065 (C)	1.569	0.061	0.004	0.824	100	0.971
Co, 0.110 (A)	1.446	0.097	0.013	0.805	88.7	0.870
Co, 0.075 (B)	1.542	0.061	0.014	0.932	95.7	0.987
Co, 0.065 (C)	1.571	0.059	0.006	0.936	80.7	0.768

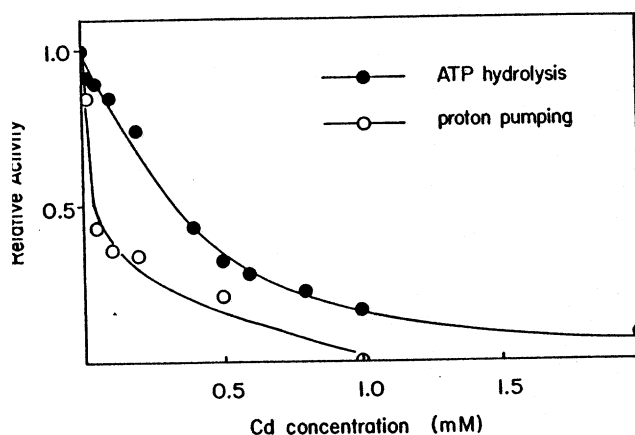


Figure 3. Effects of Cd concentration on the activities of the tonoplast ATPase. The activities were assayed using 2.5 mM Mg and 2 mM ATP as substrate. Activities are relative to the control values of 301 nmol of $\text{P}_i/\text{min}/\text{mg}$ of protein and 2.12 $\Delta A/\text{min}/\text{mg}$ of protein for ATP hydrolysis and proton pumping, respectively.

was also clear from Figure 4 that Mg-ATP was more effective in decreasing Cd inhibition of proton pumping than to ATP hydrolysis.

Effects of Divalent Cation on Membrane Structure

To a certain extent, the observed effects of free divalent cations as depicted in Tables I, II, and III, may be attributable to the binding with the phospholipids of the membrane vesicles. The binding of divalent cations to negatively charged head groups would cause a change in the membrane fluidity which may be qualitatively assessed by the rotational freedom of incorporated, lipid-soluble fluorescent probes, *e.g.* DPH (18). The quantum yield of the fluorescence of DPH is determined by the polarity of its environment, *i.e.* nonfluorescent in aqueous media but highly fluorescent in hydrocarbon environment. A measurement of the polarization of incor-

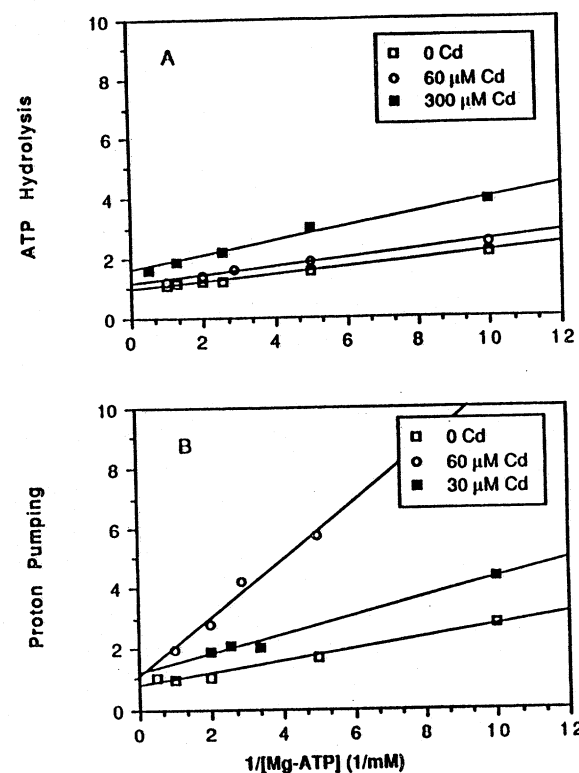


Figure 4. Effects of Cd on the kinetics of the activities of the tonoplast ATPase. The effects of Mg-ATP concentration on the inhibition induced by 0 to 300 μM Cd^{2+} were evaluated. Plots of $1/V$ (relative scale) versus $1/S$ revealed a complex inhibition pattern for both ATP hydrolysis (A) and proton pumping (B). Sigma Plot software was used for linear regression analysis.

porated DPH under different conditions would yield needed information to assess the structural change of the membrane. As shown in Table IV, the fluorescence polarization of DPH in the tonoplast membrane was only slightly affected by th

Table IV. Fluorescence Quenching and Polarization of Incorporated DPH

Divalent Cation ^a	Relative Intensity (<i>I</i> ₁)	Polarization
None	100 ^b	0.180
Mg ²⁺	94	0.189
Ca ²⁺	97	0.193
Ba ²⁺	83	0.190
Cd ²⁺	72	0.195
Zn ²⁺	86	0.192
Cu ²⁺	58	0.203
Mn ²⁺	68	0.194

^a Concentration of cation added was 0.200 mM. ^b Error in fluorescence intensity measurement was $\pm 2\%$.

presence of divalent cations. In general, the tested cations increased the polarization by no more than 10%, indicating a slight increase in the rigidity of the membrane. This suggested that, under the employed experimental conditions, the binding of cations to the negatively charged polar head groups of phospholipids in the membrane did not significantly change the microenvironment of DPH to affect its rotational motion. However, the fluorescence intensity of DPH was more noticeably affected. Thus, the structural change of the lipid phase induced by the binding of cations was sufficient to affect its polarity as detected by quantum yield measurement. It should be mentioned that Cu²⁺ and Mn²⁺ did not change the absorption of the vesicle suspension around 448 nm. Therefore, the observed changes in intensity were not due to the trivial reabsorption of emitted fluorescence or "inner filtering effect." In addition to the likely interactions of M-ATP at the catalytic and regulatory site(s) of the enzymes, these detected structural changes caused by free cations in the lipid phase could also contribute to the inhibition described in Tables I, II, and III.

DISCUSSION

In agreement with previous reports (7, 8), the preference for Mg-ATP as the substrate to support the coupled ATP hydrolysis and proton pumping by the tonoplast H⁺-ATPase is now firmly established for corn root system. The presence of other divalent cations, with the exception of Ca²⁺ and Ba²⁺, inhibited both portions of the coupled process. Under the experimental conditions, the inhibitory cations (Cu, Co, Zn, and Cd) existed in both ATP-complexed and free forms. Since the dissociation constants of these ATP complexes (Cu, Co, and Zn) are lower than that of Mg-ATP, only a minor portion of the cations exists in free forms. Thus, it is expected that the ATP complexes are involved in the inhibition of the enzyme. The observed inhibition of Mg-ATP hydrolysis by Zn-ATP and Cd-ATP (Tables II and III) and of proton pumping by all ATP complexes of Class I cations (Table III) are examples.

Presumably, those complexes may bind to the active site of the enzyme. Since the cation complexes themselves do not significantly support the coupled activities, the simple competitive model predicts that the activities will be decreased due to the lowered concentration of the enzyme-Mg-ATP complex. Since the decrease of ATP hydrolysis rate was considerably less than that of the proton pumping (Tables I,

II, and III), the binding of inhibitory complexes exerted an additional effect on the function of the tonoplast H⁺-ATPase, other than a simple dilution of active enzyme-substrate complex. A likely source of this effect could be attributed to the presence of regulatory nucleotide binding site(s), as suggested by the observed inhibition of tonoplast ATPase activity at higher concentrations of Mg-ATP (21). It is possible that the inhibitory cation-ATP complexes mentioned in this work may have a higher affinity than Mg-ATP to those sites, and that the binding at the regulatory site(s) might preferentially affect the proton pumping pathway.

Although only a small portion of the inhibitory cations exists in free form, the present study nevertheless indicates that their possible roles in either contributing to or regulating the observed inhibition should not be overlooked. For example, as shown in Tables I and II, an increase in the concentration of free Co²⁺ or Cu²⁺ leads to a greater inhibition of ATP hydrolysis. The possible regulatory effects of these two cations on the proton pumping process are suggested by the results in Table III. An increase in their concentrations resulting in less inhibition suggests that these free cations may either displace the complexes or bind to some other region to weaken the effect of the complexes. In either case, the binding of these free cations does not necessarily lead to inhibition. On the other hand, free Zn²⁺ appears to be able to inhibit proton pumping directly.

Mechanistically, the observed differential inhibition of the coupled activities of the tonoplast H⁺-ATPase caused by the cations and their ATP-complexes may be accounted for by our previously proposed "indirect" coupling model (28). We assume that two functionally distinctive peptide domains, the ATP hydrolysis domain and the protogenic domain, together with a linkage structure constitute the essential elements for the coupled activities of the tonoplast H⁺-ATPase. The energy released from Mg-ATP hydrolysis may be first conserved in the enzyme (E, the ATPase) structure as conformational strains, *i.e.* changing from E to an activated form (E*). The E* can then relax back to E by transmitting this energy to the protogenic domain to induce the vectorial proton translocation. Thus, the results of Tables I, II, and III and Figure 3 would suggest the binding of the inhibitory divalent cations and their ATP complexes to the enzyme might result in preventing the formation or in changing the relaxation or both, of the proper E*.

Because the initial rates of proton pumping (*R*₀) described in this work were calculated from the leakage constant (*k*₁) and the extent of proton translocation at steady state (δ_s), the risk of acquiring subjective errors from drawing tangent lines through the initial portion of pumping curves was minimized. Furthermore, the mathematic model applied in this work was also substantiated by the independent works of other researchers (10). Since the change in proton pumping caused by the divalent cations (except Ba²⁺ and Ca²⁺) and their ATP complexes was not accompanied by any significant change in the leakage constant (*k*₁), the observed inhibition could not be the consequence of enhanced H⁺ leakage or unspecified secondary ion transport. Thus, the results reported in this work together with our previously described differential effects of nitrate (27) and mercuric ions (28) supported the operation

of an "indirect" coupling mechanism in the tonoplast H⁺-ATPase of corn roots.

LITERATURE CITED

1. Bennett AB, O'Neill SD, Spanswick RM (1984) H⁺-ATPase activity of storage tissue of *Beta vulgaris*. I. Identification and characterization of an anion H⁺-ATPase. *Plant Physiol* 74: 538-544
2. Bennett AB, Spanswick RM (1984) H⁺-ATPase activity from storage tissue of *Beta vulgaris*. *Plant Physiol* 74: 545-548
3. Bennett AB, Spanswick RM (1983) Optical measurement of ΔpH and $\Delta\Psi$ in corn root membrane vesicles: Kinetic analysis of Cl⁻ effects on a proton translocating ATPase. *J Membr Biol* 71: 95-107
4. Bowman EJ, Mandala S, Taiz L, Bowman BJ (1986) Structural studies of the vacuolar membrane ATPase from *Neurospora crassa* and comparison with the tonoplast membrane ATPase from *Zea mays*. *Proc Natl Acad Sci USA* 83: 48-52
5. Briskin DP, Leonard RT (1982) Partial characterization of a phosphorylated intermediate associated with the plasma membrane ATPase of corn roots. *Proc Natl Acad Sci USA* 79: 6922-6926
6. Canson A, Fichmann J, Spear D, Taiz L (1985) Pyrophosphate-driven proton transport by microsomal membranes of corn coleoptiles. *Plant Physiol* 79: 159-164
7. Churchill KA, Sze H (1983) Anion-sensitive, H⁺-pumping ATPase in membrane vesicles from oat roots. *Plant Physiol* 71: 610-617
8. Du Pont FM, Giorgi DL, Spanswick RM (1982) Characterization of a proton-translocating ATPase in microsomal vesicles from corn roots. *Plant Physiol* 70: 1694-1699
9. Ho YK, Liu J, Saunders DR, Wang JH (1979) Light dependence of the decay of the proton gradient in broken chloroplasts. *Biochim Biophys Acta* 547: 149-160
10. Jennings IR, Rea PA, Leigh RA, Sanders D (1988) Quantitative and rapid estimation of H⁺ fluxes in membrane vesicles: Software for analysis of fluorescence quenching and relaxation. *Plant Physiol* 86: 1257-1263
11. Mandala S, Taiz L (1986) Characterization of the subunit structure of the maize tonoplast ATPase. *J Biol Chem* 261: 12850-12855
12. Mitchell P (1975) Vectorial chemistry and the molecular mechanism of chemiosmotic coupling: Power transmission by proticity. *FEBS Lett* 59: 137-139
13. Pietrobon D, Zoratti M, Azzone GF, Stucki JW, Walz D (1982) Non-equilibrium thermodynamic assessment of redox-driven H⁺ pumps in mitochondria. *Eur J Biochem* 127: 483-494
14. Pope AJ, Leigh RA (1987) Some characteristics of anion transport at the tonoplast of oat roots, determined from the effects of anions on pyrophosphate-dependent proton transport. *Planta* 172: 91-100
15. Scalla R, Amory A, Rigand J, Goffeau A (1983) Phosphorylated intermediate of a transport ATPase and activity of protein kinase in membranes from corn roots. *Eur J Biochem* 132: 525-530
16. Schumaker KS, Sze H (1986) Calcium transport into the vacuole of oat roots. *J Biol Chem* 261: 12172-12178
17. Schumaker KS, Sze H (1987) Decrease of pH gradients in tonoplast vesicles by NO₃⁻ and Cl⁻: Evidence for H⁺-coupled anion transport. *Plant Physiol* 83: 490-496
18. Shinitzky M, Inbar M (1976) Microviscosity parameters and protein mobility in biological membranes. *Biochim Biophys Acta* 433: 133-149
19. Sze H (1985) H⁺-translocating ATPases: Advances using membrane vesicles. *Annu Rev Plant Physiol* 36: 175-208
20. Taqui Khan MM, Martell AE (1966) Metal chelates of adenosine diphosphoric and adenosine-monophosphoric acids. *J Am Chem Soc* 84: 3037-3041
21. Thom M, Komor E (1984) Effects of magnesium and ATP on ATPase of sugarcane vacuoles. *Planta* 161: 361-365
22. Ting LP, Wang JH (1982) Efficiency of proton extrusion by chemically modified mitochondria. *Biochim Biophys Acta* 682: 474-481
23. Tu S-I, Hutchinson H (1984) Temperature dependence of light-induced proton movement in reconstituted purple membrane. *Arch Biochem Biophys* 228: 609-616
24. Tu S-I, Sliwinski BJ (1985) Mechanistic investigation on the temperature dependence and inhibition of corn root plasma membrane ATPase. *Arch Biochem Biophys* 241: 348-355
25. Tu S-I, Lam E, Ramirez F, Marecek JF (1981) Inhibition of the links between electron transfer and proton translocation in mitochondria. *Eur J Biochem* 113: 391-396
26. Tu S-I, Nagahashi G, Sliwinski BJ (1984) Regulatory effects of adenosine diphosphate on the activity of the plasma membrane ATPase of corn roots. *Biochem Biophys Res Commun* 122: 1367-1373
27. Tu S-I, Nagahashi G, Brouillette JN (1987) Proton pumping kinetics and origin of nitrate inhibition of tonoplast-type H⁺-ATPase. *Arch Biochem Biophys* 256: 625-637
28. Tu S-I, Brouillette JN, Nagahashi G, Brauer D, Nungesser E (1988) Temperature dependence and mercury inhibition of tonoplast-type H⁺-ATPase. *Arch Biochem Biophys* 266: 289-297
29. Walker RR, Leigh RA (1981) Characterization of a salt-stimulated ATPase activity associated with vacuoles isolated from storage roots of red beet (*Beta vulgaris* L.). *Planta* 153: 140-149
30. Westerhoff HV, Hellingwerf KJ, Arents JC, Scholte BJ, Van Dam K (1982) Mosaic non-equilibrium thermodynamics describes biological energy transduction. *Proc Natl Acad Sci USA* 78: 3554-3558